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A study of the characteristics of "P" and "Q" strains of *Trichoderma virens* to account for differences in biological control efficacy against cotton seedling diseases

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Abstract

Strains of *Trichoderma virens* belonging to the "P" group are ineffective as biocontrol agents of seedling disease in cotton, and they are pathogenic to susceptible seed lots. Those strains belonging to the "Q" group are effective biocontrol agents of cotton seedling disease, and they are not pathogenic to cotton. To account for these behavioral differences, comparative assays were made of "P" and "Q" strains for production of phytotoxin, for cellulase, polygalacturonase and protease activity, for induction of phytoalexin synthesis in cotton roots, and for metabolism of pathogen germination stimulants. The results showed little difference in phyotoxin production or enzyme activity between the two groups, and that "P" strain mutants deficient for viridiol production were still pathogenic to cotton. There was also no difference between strains in their ability to metabolize pathogen germination stimulants. HPLC analyses of extracts from roots treated with "P" or "Q" strains, however, showed that "Q" strains induced high levels of phytoalexin synthesis, while "P" strains did not. Treatment of seeds or seedling radicles with combination "P" + "Q" seed coat preparations or cultures filtrates, respectively, ameliorated seedling kill, and increased phytoalexin production in treated roots. These results indicate that an inability by "P" strains to induce high levels of phytoalexins in cotton, not only makes them ineffective as biocontrol agents, but renders them pathogenic to susceptible cultivar seed lots. Induction by "Q" strains of high levels of phytoalexin synthesis in cotton makes them effective biocontrol agents, and it inhibits their development in cotton roots which might lead to pathogenesis.
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Keywords: *Trichoderma virens*; Germination stimulants; Cellulase; Polygalacturonase; Protease; Phytotoxin; Phytoalexins; Pathogenicity

1. Introduction

The imperfect fungus, *Trichoderma virens* (Miller, Giddens and Foster) von Arx, Beih., has been demonstrated on many occasions to be an effective biocontrol agent of seedling and root diseases on a number of crops (Beagle-Ristaino and Papavizas, 1985; Howell, 1982, 1991; Howell et al., 1997; Lumsden and Locke, 1989; Tu and Vaartaja, 1981). Strains of the biocontrol fungus *T. virens* can be separated into two distinct groups on the basis of their antibiotic production (Howell et al., 1993). The "Q" strains of *T. virens* produce the wide spectrum diketopiperazine antibiotic gliotoxin. The "P" strains do not produce gliotoxin, but they do produce a closely related compound, gliovirin, whose activity spectrum is confined to the Oomycetes (Howell and Stipanovic, 1983). Strains of *T. virens* also synthesize the steroid viridiol on low nitrogen containing substrates (Howell and Stipanovic, 1984; Jones and Hancock, 1987). Viridiol is a potent phytotoxin when applied to roots or seeds, and seed treatment with *T. virens* preparations containing high levels of the compound results in dead plants. Later studies on the mechanisms employed by *T. virens* to effect biological control have indicated that antibiotic production by this fungus has little or nothing to do with

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effective biocontrol (Howell and Stipanovic, 1995). The most important mechanisms in the biocontrol of cotton seedling diseases by this fungus appear to be plant phytoalexin induction and metabolism of pathogen germination stimulants produced by germinating cotton seedlings (Howell et al., 2000; Howell, 2002). Preliminary comparative studies on the efficacy of "P" and "Q" strains of *T. virens* as biocontrol agents of cotton seedling disease have indicated that "Q" strains give very effective disease control, while "P" strains do not. The purpose of this research was to determine what characteristics of "P" and "Q" strains of *T. virens* could account for the difference in their biocontrol efficacies. Answers to these questions should help to further elucidate what mechanisms are most important in the biological control process, and they may help to determine what characteristics might best be enhanced to optimize disease control by this fungus.

2. Materials and methods

2.1. Maintenance and culture of microbial isolates

Trichoderma virens "Q" strains G-6, Ga6-3, Fl7-6 and Al5-4 and "P" strains G-4, G-9, Ga6-4, and NC16-1 were maintained on PDA plates containing 50 μg ml^{-1} rifampicin until used. Biocontrol preparations of each strain were produced as shake cultures in a medium consisting of 5 g wheat bran, 1 g peat moss and 100 ml water, adjusted to pH 4.0 with HCl. The cultures were incubated at 27 °C and 150 rpm for 6 days before harvest, and then they were centrifuged at 5000 rpm for 10 min. The supernates were decanted and stored in the freezer, and the solids were spread in sterile petri dishes under a positive pressure hood to dry overnight. The air-dried solids were then placed in Ziploc bags and stored in the refrigerator at 5 °C until used.

2.2. Bioassay of "P" and "Q" strain preparations for pathogenicity to cotton seedlings

Susceptible seed lots of the cotton cultivar Deltapine 451 B/RR were coated with fine granules of the air-dried culture solids of *T. virens* "P" and "Q" strains, described in Section 2.1, after coating the seed with a latex sticker. The seeds were then planted in test tubes containing 10 g moist soil that was not infested with cotton seedling disease pathogens. This soil was the same Lufkin fine sandy loam found in the seedling disease plots, except that it had never been planted with crops. Each treatment, consisting of five tubes, was replicated three times, and the experiment was repeated twice. After 7 days incubation in a growth chamber at 25 °C and with a 14 h photoperiod, the numbers of surviving seedlings were counted. Seeds and seedlings that did not emerge in non-infested soil were harvested, surface sterilized with 1% sodium hypochlorite and 70% ethanol, then washed in sterile water and the embryos plated on PDA containing 50 μg ml^{-1} rifampicin to observe for fungal growth.

2.3. Comparison of "P" and "Q" strains for viridiol production and cellulase, polygalacturonase, and protease activities

2.3.1. Viridiol

The "Q" strain G-6, "P" strain G-4 and viridiol-deficient mutants G4-10V and G4-21V were shake incubated in 100 ml cultures containing 5% ground millet. Cultures were incubated for 6 days at 25 °C, and then the supernates were each extracted twice with 50 ml volumes of chloroform. The chloroform extracts were taken to dryness and dissolved in 2 ml volumes of methanol. Aliquants of the extracts were subjected to HPLC analysis as described previously (Howell et al., 1993), and the viridiol concentrations in the "Q," "P" and viridiol-deficient strains were compared. The pathogenicities of "P" strains G-4, G4-10V, and G4-21V to the cotton cultivar DP451B/RR were also assayed as described in Section 2.2.

2.3.2. Cellulase assay

An agar gel containing 1.7% agar and 0.5% carboxymethylcellulose was prepared, autoclaved, and poured in 15 ml lots into petri dishes. Three 7 mm diameter wells were cut into each plate with a cork borer, and each well was filled with 80 μl of supernates from 6 day shake cultures (5% wheat bran + 1% peat moss) of "Q" strains G-6, Fl7-6 and Al5-4, and "P" strains G-4, G-9, and NC16-1. The wells in control plates were filled with heat killed culture filtrates. After 48 h at 30 °C, the plates were flooded with 2 ml congo red dye (1 mg ml^{-1}). The dye was decanted after 15 min, and the plates were washed with 1 M NaCl. The plates were then observed for the presence of clear zones around the wells. Three replicates were made for each treatment, and the experiment was repeated twice.

2.3.3. Polygalacturonase assay

An assay medium consisting of Raulin-Thom agar medium (Raper and Thom, 1949) containing 0.5% sodium polygalcturonate was prepared, adjusted to pH 5.0, and amended with 0.3 μg ml^{-1} benomyl. The medium was autoclaved and 15 ml aliquants were poured into petri dishes. The plates were inoculated with three 4 mm plugs each of "Q" strains G-6, Fl7-6 and Al5-4, and "P" strains G-4, G-9 and NC16-1. After 72 h incubation at 27 °C, the plates were flooded with 1% hexadecyltrimethylammonium bromide. After 10 min, the excess was decanted and the plates observed for clear zones around the colonies.

2.3.4. Protease assay

An assay medium consisting of 1.6% agar, and 0.4% gelatin in 0.2 M phosphate buffer was prepared and adjusted to pH 6.0. The medium was autoclaved, and 15 ml plates were poured. Wells were cut into the plates and filled with culture filtrates of the "Q" strains G-6, Fl7-6 and Al5-4, and the "P" strains G-4, G-9 and NC16-1 as described above. The wells in control plates were filled with heat killed culture filtrates. After 48 h incubation at 27 °C, the plates were flooded with 10% trichloroacetic acid. After 5 min, the excess was decanted and the plates were observed for the presence of clear zones around the wells.

2.4. Assay of "P" and "Q" strain culture filtrates for induction of phytoalexin synthesis in cotton seedling radicles

The filtrates from 6 day wheat bran + peat moss shake cultures of "P" strains G-4, G-9 and NC16-1, and "Q" strains G-6, Al5-4 and Fl7-6 of *T. virens* were prepared as described in Section 2.1 and applied to the radicles of the cotton cultivar DP451 B/RR 48 hrs after germination. After 24 h incubation at 27 °C, the radicles were harvested, extracted and analyzed as described in Howell et al. (2000).

2.5. Comparison of "P" and "Q" strains for metabolism of pathogen propagule germination stimulants

Shake cultures (50 ml in 125 ml flasks) of Raulin-Thom's medium (Raper and Thom, 1949) were inoculated with "P" strains G-4 or G-9, or "Q" strains G-6 or Al5-4 and incubated for 3 days at 27 °C and 150 rpm. On day 3, 1 ml aliquants of 20× pathogen propagule germination stimulants, that had been isolated from cotton seed exudates, concentrated, and partially purified, were added to the cultures. Germination stimulants had been partially purified by extraction of the seed exudates with ethyl acetate, followed by precipitation of the stimulants from the aqueous residue with absolute ethanol. The precipitated stimulants were dissolved in water and further purified by passage through cation and anion exchange columns before concentration by freeze drying. After 48 h shake incubation, culture solids were removed by centrifugation at 3000 rpm, and 20 ml aliquants of the supernates were mixed with 80 ml acetone. The mixtures were centrifuged at 4500 rpm for 25 min, and the solution was decanted. The remaining solids were air dried to remove residual acetone, and then dissolved in 1 ml aliquants of sterile deionized water. Aliquants of the aqueous residues left after acetone precipitation were added to *Rhizopus oryzae* sporangiospores (1×10^6 spores ml^{-1}) at a ratio of two parts to one, and three aliquants (50 µl) of each sample were spotted on Noble agar plates. After 24 h at 25 °C, the cultures were observed for evidence of spore germination and mycelial growth. The control was a non-inoculated Raulin-Thom culture with added propagule germination stimulant precipitated and assayed as described above.

2.6. Effect of combined "P" and "Q" strain seed treatments on seedling emergence and effect of combined culture filtrate application to seedling radicles on induction of phytoalexin synthesis

2.6.1. Seed treatments

Cotton seed of the cultivar DP451 B/RR were coated with latex and air-dried preparations of "P" and "Q" strains. The seeds were planted in non-infested soil tubes as described in Section 2.2, except that combination "P" and "Q" treatments were applied. Combined treatments were G-6 + G-4, Al5-4 + G-9, Fl7-6 + NC16-1 and Ga6-3 + Ga6-4. Strains G-4, G-9, Ga6-4, and NC16-1 were combined with equal weights of killed culture preparation prior to seed treatment, and seeds were coated with killed culture preparation as controls. After 7 days incubation at 25 °C, the tubes containing emerged and non-emerged seedlings were counted.

2.6.2. Culture filtrate treatment of seedling radicles

The radicles of DP451 B/RR seedlings were treated with culture filtrate as described in Section 2.4, except that the treatments were combined equal volumes of "P" and "Q" culture filtrates. The combinations were G-6 + G-4, Al5-4 + G-9, Fl7-6 + NC16-1, and Ga6-3 + Ga6-4. The controls were treated with culture filtrate (diluted 1:1 with killed culture filtrate) from strains G-4, G-9, NC16-1, or Ga6-4. After 24 h, the radicles were harvested, extracted, and analyzed as previously described in Howell et al. (2000).

2.7. Statistical analysis

All treatments in the experiments were replicated a minimum of three times, and all experiments were repeated twice with similar results. The data were analyzed using general linear models (version 6; SAS Institute, Cary, NC), and the datasets were analyzed individually.

3. Results

3.1. Bioassay of "P" and "Q" strain preparations for biocontrol efficacy and pathogenicity

The data on survival of cotton seedlings from seeds treated with "P" and "Q" strain preparations of *T. virens* and planted in pathogen infested soil had indicated that "P" strains were ineffective as biocontrol agents of cotton seedling disease (data not presented). However, the

Table 1
Bioassay of "P" and "Q" strains of *T. virens* for pathogenicity to *T. virens* treated cotton seedlings planted in non-infested field soil

Seed treatment[A]	% seedling survival[B]
NT	80[a]
G-6 (Q)	87[a]
Fl7-6 (Q)	80[a]
Al5-4 (Q)	93[a]
Ga6-3 (Q)	87[a]
G-4 (P)	27[bc]
G-9 (P)	27[bc]
Ga6-4 (P)	40[b]
NC16-1 (P)	20[c]

[A] DeltaPine 451 B/RR cotton seed were coated with latex sticker and air-dried granules from wheat bran + peat moss cultures of *T. virens*. NT, non-treated; (P), "P" strains and (Q), "Q" strains.
[B] Data are the averages of three replications. Means in the column followed by different letters are significantly different according to the protected least significant difference test at $\alpha = 0.05$, using general linear models (SAS Institute, Cary, NC).

data derived from "P" strain treated seeds planted in non-infested soil indicated that the "P" strains themselves were pathogenic to cotton seedlings (Table 1). Seeds treated with "Q" strain preparations produced a high percentage of emerged and healthy seedlings, while most of those treated with "P" strains did not emerge. Embryos, harvested from "P" strain treated seeds that did not emerge from non-infested soil, and were surface sterilized and plated on antibiotic PDA, produced pure cultures of *T. virens* "P" strains.

3.2. Assay of "P" and "Q" strains for viridiol synthesis and pathogenicity of viridiol-deficient "P" strain mutants

A comparison of "P" strains, "Q" strains, and viridiol-deficient "P" strains for viridiol production and pathogenicity to cotton seedlings showed that the parent strains of both groups produced viridiol on a medium that promotes its production. The "Q"strain G-6 produced 47.2 $\mu g\,ml^{-1}$ of viridiol, while the "P"strain G-4 produced 44 $\mu g\,ml^{-1}$. The viridiol-deficient "P" strains G4-10V and G4-21V produced no viridiol in culture. The "Q" strain G-6 grown on the medium was not pathogenic (93% seedling survival), while the "P" strain G-4 (33% seedling survival) and the viridiol-deficient mutants G4-10V (27% seedling survival) and G4-21V (27% seedling survival) were pathogenic to cotton seedlings.

3.3. Assay of "P" and "Q" strains for lytic enzyme activity

Assays of "P" and "Q" strains for cellulase, polygalacturonase, and protease activity showed that both groups produced clear zones in the assay media, indicating that both were capable of synthesizing these enzymes. Control plates treated with heat killed culture filtrates produced no clear zones in the media.

Table 2
Assay of "P" and "Q" strain culture filtrates of *Trichoderma virens* for induction of phytoalexins (hemigossypol and desoxyhemigossypol) synthesis in cotton seedling radicles

Strain[A]	Phytoalexins (μg/g tissue)[B]
Al5-4 (Q)	115.3[a]
Fl7-6 (Q)	114.8[a]
G-6 (Q)	101.8[a]
Ga6-3 (Q)	85.5[ab]
G-4 (P)	52.9[c]
Ga6-4 (P)	49.0[c]
G-9 (P)	47.1[c]
NC16-1 (P)	24.9[d]
Control (H_2O)	4.5[e]

[A] Radicles of DeltaPine 451 B/RR were treated with wheat bran + peat moss culture filtrates of *T. virens*, and radicle extracts were analyzed by HPLC to determine phytoalexin concentrations. (P), "P" strains and (Q), "Q" strains of *T. virens*.
[B] Data are the averages of three replications. Means followed by different letters are significantly different according to the protected least significant difference test at $\alpha = 0.05$, using general linear models (SAS Institute, Cary, NC).

3.4. Assay of "P" and "Q" strains for induction of phytoalexin synthesis in cotton radicles

HPLC analyses of extracts of cotton seedling radicles that were treated with "P" or "Q" strain culture filtrates, showed that "P" strains induced only low levels of the terpenoid phytoalexins hemigossypol and desoxyhemigossypol in root tissue. Cotton radicles treated with "Q" strain filtrates synthesized significantly higher concentrations of the two terpenoid phytoalexins (Table 2).

3.5. Comparison of "P" and "Q" strains for metabolism of pathogen propagule germination stimulants

Examination of Noble agar plates spotted with mixtures of *R. oryzae* spores and the aqueous residues from acetone precipitated culture filtrates of "P" and "Q" strains containing pathogen propagule germination stimulants, showed that spores mixed with the residue from the non-inoculated control germinated and grew. Those spores mixed with the residues from "P" and "Q" strain cultures did not. These results indicate that both "P" and "Q" strains are capable of metabolizing pathogen germination stimulants excreted by seeds. Spores mixed with "P" and "Q" strain cultures and applied to PDA did germinate and grow, indicating that failure to germinate on Noble agar was not due to the presence of inhibitors in the culture residues.

3.6. Effect of combined "P" and "Q" seed treatments on seedling survival and combined culture filtrate application to radicles on phytoalexin synthesis

The results of cotton seed treatments with combinations of "P" and "Q" strain air-dried preparations and

Table 3
Effect of "P" and combined "P" and "Q" strain seed treatments and culture filtrate applications to radicles on seedling survival and induction of phytoalexin synthesis, respectively

Strains[A]	% survival[B]	Phytoalexins (μg/g tissue)[C]
NT control	93[a]	—
G-6 (Q) + G-4 (P)	87[a]	71.6[a]
Al5-4 (Q) + G-9 (P)	73[b]	70.4[a]
Fl7-6 (Q) + NC16-1 (P)	73[b]	58.7[b]
G-4 (P)	27[c]	44.6[c]
G-9 (P)	27[c]	22.9[d]
NC16-1 (P)	27[c]	31.1[d]

[A] DeltaPine 451 B/RR seed were treated with wheat bran + peat moss culture granules of *T. virens* and planted in non-infested soil. Radicles were treated with filtrates from these cultures. "P" strain cultures G-4, G-9, and NC16-1 were diluted to half strength with heat killed wheat bran + peat moss cultures before seed treatment. Combination treatments were diluted with equal amounts of the "Q" strains.

[B] Data are the averages of three replications. Means followed by different letters are significantly different according to the protected least significant test at $\alpha = 0.05$, using general linear models (SAS Institute, Cary, NC).

[C] Phytoalexin (hemigossypol + desoxyhemigossypol) concentrations induced by culture filtrates in cotton radicles are the means of three replications and were statistically analyzed as described above.

planted in non-infested soil showed that pathogenicity of the "P" strain components was ameliorated by the presence of the "Q" strains (Table 3).

This was also the case where seedling radicles were treated with combinations of "P" and "Q" strain culture filtrates. The induction of terpenoid phytoalexins in cotton roots by "P" strain filtrates was considerably enhanced by the presence of culture filtrate from "Q" strains (Table 3). Dilution of "P" strain preparations and culture filtrates with heat killed preparations or culture filtrates of "Q" strains did not enhance seedling survival or phytoalexin synthesis.

4. Conclusions

The initial results derived from seed treatments of cotton seeds with "P" and "Q" strains of *T. virens* and planted in pathogen infested soil indicated that "Q" strains controlled damping-off, while "P" strains were ineffective as seedling disease biocontrol agents. However, planting "P" and "Q" strain treated cotton seeds in non-infested soil showed that "P" strains were not just ineffective in disease control, they were pathogenic to cotton seedlings. The "Q' strains were not pathogenic to cotton. These data led to several hypotheses to explain the differences observed in pathogenicity between "P" and "Q" strains. The first hypothesis was that "P" strains were producing the phytotoxin viridiol in the spermosphere, whereas the "Q" strains were not. However, assay of "P" and "Q" strains for viridiol production, and assay of "P" strain viridiol-deficient mutants for pathogenicity to cotton seedlings showed that both groups were equally capable of producing viridiol and that viridiol-deficient mutants of "P" strains still retained pathogenicity to cotton. Therefore, viridiol was not involved in the disease syndrome. The second hypothesis was that "P" strains synthesized more of the lytic enzymes cellulase, polygalacturonase and protease that are thought to be involved in the destruction of host plant tissue (Griffin, 1994), than did "Q" strains. Assay of "P" and "Q" strains for enzyme activity, however, indicated that both groups were capable of synthesizing these enzymes. Therefore, the reason for the observed differences must lie elsewhere.

The third hypothesis was that "Q" strains were capable of metabolizing the pathogen germination stimulants, produced by germinating seeds, before they affected pathogen resting structures in the soil. The "P" strains were ineffective as biocontrol agents because they could not metabolize these stimulatory compounds. However, the results of the assays for the presence of germination stimulatory compounds in culture filtrates of "P" and "Q" strains, to which they were added, indicated that both groups were equally capable of metabolizing these compounds.

The fourth hypothesis was that "P" and "Q" strains differed in their capacity to induce phytoalexin induction in cotton roots. That, although both groups are low grade pathogens, as evidenced by their penetration and colonization of the root epidermis and cortex (Howell et al., 2000; Yedidia et al., 1999), further development of "Q" strains in root tissue was inhibited by their induction of high levels of phytoalexins. Further development of low level phytoalexin inducing "P" strains was not retarded, and pathogenesis occurred. The data derived from HPLC analyses of the cotton seedling radicles treated with culture filtrates from "P" and "Q" strains indicated that this was indeed the case. Treatment of cotton radicles with "P" strain filtrates induced only low levels of terpenoid phytoalexin synthesis in roots, while treatment with "Q" strain filtrates induced much higher levels of phytoalexin synthesis. Surface sterilized embryos taken from non-emerged soil tubes containing "P" strain treated seed and plated on antibiotic PDA had pure cultures of *T. virens* "P" strains growing from them.

The fourth hypothesis is further supported by the results of assays of "P" and "P" + "Q" strain combination seed treatments for pathogenesis to cotton, and a comparison of "P", and "P" + "Q" combination culture filtrates for induction of phytoalexins in seedling radicles. Seed treatment with "P" strain preparations diluted to half strength with heat killed wheat bran + peat moss culture solids still killed most of the seedlings before emergence, while "P" strain preparations diluted with live "Q" strain preparations did not. Treatment of cotton radicles with "P" strain culture filtrates diluted to half strength with heat killed culture filtrates elicited

only minor synthesis of terpenoid phytoalexins, while filtrates diluted with "Q" strain filtrates induced significantly higher levels of phytoalexin synthesis.

The results of these experiments all support the idea that induction of phytoalexin synthesis in cotton roots by biocontrol strains of *T. virens* is an important mechanism in the defense of susceptible cotton cultivars to seedling disease, and that this phenomenon not only protects the plant from subsequent infection by pathogens, but it also protects the plant from pathogenesis by the biocontrol agent itself.

Differential phytoalexin induction in plants by "P" and "Q" strains may be due to differences in their capacity to synthesize the protein known to induce phytoalexin production (Hanson and Howell, 2004). Cloning of the gene coding for this protein and its transformation into different strains or species with other beneficial characteristics may help to optimize biological control of seedling diseases.

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